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Spermidine mitigates wheat copper toxicity by modulating ascorbate and glutathione metabolism, copper accumulation and photosynthetic performance

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Abstract: The influence of spermidine (Spd) on wheat ascorbate and glutathione metabolism, copper (Cu) accumulation and photosynthetic performance under Cu stress was studied. The findings displayed that Cu stress boosted reduced ascorbate (AsA) and reduced glutathione (GSH) contents by improving ascorbate peroxidase (APX), glutathione reductase (GR), dehydroascorbate reductase (DHAR), monodehydroascorbate reductase (MDHAR), L-galactono-1,4-lactone dehydrogenase (GalLDH) and gamma-glutamylcysteine synthetase (γ-ECS) activities. Nevertheless, Cu stress promoted malondialdehyde (MDA) accumulation and electrolyte leakage (EL) level, and lowered AsA/dehydroascorbic acid (DHA) and GSH/oxidised glutathione (GSSG). Meanwhile, Cu stress promoted Cu accumulation in plant tissues. It declined net photosynthetic rate (P_n), chlorophyll fluorescence parameter maximum photochemical efficiency of PSII (F_v/F_m), chlorophyll (*Chl*) and carotenoids (Car) contents, and wheat height and biomass. In this way, Cu stresses limited wheat growth. Compared with Cu stress, Spd plus Cu stress enhanced APX, GR, DHAR, MDHAR, GalLDH and γ-ECS activities to 4.75, 5.14, 3.77, 2.96, 3.24 and 2.83 U/g FW (fresh weight), respectively. This way, Spd further increased AsA and GSH contents to 4.62 and 0.78 μmol/g FW under Cu stress. Meanwhile, Spd increased AsA/DHA to 14.60 and GSH/GSSG to 15.97 and declined MDA content to 11.68 nmol/g FW and EL to 17.00% under Cu stress. Besides, Spd declined Cu content in leaves to 68.8 μg/g DW and roots to 152.9 μg/g DW and respectively increased P_n, F_v/F_m and *Chl* and Car contents to 15.22 μmol/m^2/s, 0.74, 1.55 mg/g FW and 0.38 mg/g FW. In this way, Spd promoted wheat growth under Cu stress. Meanwhile, we found that Spd alone also improved the ascorbate and glutathione metabolism, photosynthetic performance, and wheat growth compared to the control. These results illustrated that Spd mitigated wheat Cu toxicity by reducing Cu accumulation and improving ascorbate and glutathione metabolism and photosynthetic performance. Hence, using Spd will be a good strategy to improve the Cu tolerance of wheat crops in the future.

Keywords: environmental pollutant; micronutrient; antioxidant metabolism; non-enzymatic antioxidants; *Triticum aestivum* L.

At low concentrations, heavy metal copper (Cu) is an important mineral nutrient element for plant growth. It can act as a cofactor of enzymes and participate in photosynthesis and cell wall metabolism (Sun et al. 2022). In agriculture, Cu-containing fertilisers, fungicides and sewage sludge are used in cropping systems (Panfili et al. 2019). Therefore, the anthropic activities in agriculture promote Cu accumulation in the soil of cultivated areas. However, Cu is an important toxic environmental pollutant at high concentrations. Excess Cu usually destroys the balance of reactive oxygen species (ROS) metabolism and leads to massive accumulation of ROS (Aqeel et al. 2023). In this way, Cu induced the peroxide damage to plants. Increasing research showed that plants could decrease the level of oxidation stress caused by stresses through a non-enzymatic antioxidant system (Valentovicova et al. 2010). Reduced ascorbate (AsA)

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and glutathione (GSH) are important antioxidants. Increasing research has demonstrated that plants modulated AsA and GSH contents through their recycling pathway, called the ascorbate-glutathione (AsA-GSH) cycle. In this cycle, ascorbate peroxidase (APX) can remove one important ROS named hydrogen peroxide (H_2O_2) by using AsA as the substrate. Dehydroascorbate reductase (DHAR) can reduce dehydroascorbic acid (DHA) to monodehydroascorbic acid (MDHA). Monodehydroascorbate reductase (MDHAR) can reduce MDHA to AsA. Glutathione reductase (GR) can reduce oxidised glutathione (GSSG) to GSH. Therefore, this cycle has important roles in controlling AsA and GSH contents and removing H_2O_2, thereby decreasing the oxidation stress. Meanwhile, previous studies also manifested that plants could control AsA and GSH contents by regulating their biosynthetic pathway, in which L-galactono-1,4-lactone dehydrogenase (GalLDH) is the rate-limiting enzyme for AsA biosynthesis and gamma-glutamylcysteine synthetase (γ-ECS) is the rate-limiting enzyme for GSH biosynthesis. As demonstrated, exogenous substances could enhance plant Cu tolerance (Gaur et al. 2021, Li et al. 2022, Fardus et al. 2023). Li et al. (2022) demonstrated melatonin improved rice Cu tolerance. Gaur et al. (2021) reported that silicon (Si) and nitric oxide (NO) could enhance the Cu tolerance of mung bean seedlings. Fardus et al. (2023) found that L-glutamic acid alleviated Cu toxicity in lentils. The above research results of previous studies all clearly indicated that exogenous substances can be applied to mitigate Cu toxicity in plants. Meanwhile, previous research also demonstrated that exogenous substances could enhance plant Cu tolerance by activating ascorbate and glutathione metabolism (Zhou et al. 2018, Gaur et al. 2021). As reported, 24-epibrassinolide could mitigate Cu toxicity in grapes by raising AsA content through the modulation of the AsA-GSH cycle (Zhou et al. 2018). Gaur et al. (2021) demonstrated that Si could alleviate Cu toxicity by promoting AsA and GSH accumulation in mung bean seedlings. Thus, we can use exogenous substances to enhance plant Cu tolerance by regulating AsA and GSH accumulation *via* enzymes in their recycling and biosynthetic pathways.

Polyamines (PAs) contain two or more amino groups, including putrescine (Put), spermidine (Spd) and spermine (Spm). As demonstrated, PAs played vital roles in protecting plants against different kinds of stress, including salinity-alkalinity stress (Xu et al. 2024), water deficit (Sun et al. 2023), cadmium (Cd) stress, Cu stress (Groppa et al. 2007) and salt stress (Zhao et al. 2023). The previous research clearly illustrated that PAs could enhance plant tolerance to different kinds of stress; for Spd, previous studies showed that it reinforced plant tolerance under salt (Wu et al. 2020), drought (Yin et al. 2014) and Cd stresses (Yang et al. 2013), etc. Wheat is an important crop in China. However, Cu concentrations in 2.1% of Chinese farmland soil samples exceed the limit (Zeng et al. 2021). Therefore, wheat crops planted on Cu-contaminated soil will suffer from Cu stress. So, it is important to protect wheat crops against Cu stress through some measurements. At present, Spd is an expensive chemical. Therefore, using Spd to improve wheat Cu tolerance in production is not practical. Nevertheless, exploring how Spd regulates wheat Cu tolerance will still be interesting, as it can provide the basis for its potential use in the future, especially when its price is significantly reduced. Agami (2016) found that Spd could alleviate wheat Cu toxicity by enhancing superoxide dismutase (SOD), peroxidase (POD) and APX activities. However, the regulatory mechanism of Spd in modulating wheat ascorbate and glutathione metabolism under Cu stress has still not been fully uncovered. Besides, the Cu-induced peroxide damage is closely related to the over-accumulation of Cu in plant tissues. Meanwhile, the photosynthetic performance and growth status are plant- and plant-direct manifestations in response to Cu stress at the physiological and morphological levels. Thus, it is interesting to uncover the role of Spd in regulating the ascorbate and glutathione metabolism, photosynthetic performance, Cu accumulation and wheat growth under Cu stress, which can add more information for Spd application in alleviating wheat Cu toxicity.

For this research, we investigated the function of Spd in regulating malondialdehyde (MDA) content, electrolyte leakage (EL), the activities of enzymes in the ascorbate and glutathione metabolism, AsA and GSH contents, and AsA/DHA and GSH/GSSG in the leaves, Cu content, photosynthetic performance, and wheat height and biomass under Cu stress. This study will add new knowledge for the regulatory mechanism of Spd in decreasing the level of Cu-induced oxidation stress and supply basis for the potential application of Spd in agricultural management to control the growth of wheat crops planted in Cu-polluted soil in the future.

MATERIAL AND METHODS

Plant material and treatments. Seeds of wheat (*Triticum aestivum* L.) cv. Yuanfeng16 was supplied

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by Henan Fengyuan Seed Co. Ltd. The surface sterilisation of seeds using 5.0% sodium hypochlorite, and then seeds were washed with distilled water. Afterwards, seeds were germinated on moistened filter paper in Petri dishes and placed in the artificial climate incubator to culture wheat seedlings. Wheat growth conditions in the artificial climate incubator were controlled at 25 °C (day)/18 °C (night), 500 μmol/m^2/s photosynthetic active radiation and a 10-h photoperiod. After the first leaves were fully unfolded, all seedlings' roots were submerged in 4.0 L half-strength Hoagland's nutrient solution in plastic boxes (30.0 cm length × 20.0 cm width × 10.5 cm height) and kept in the dark. According to Pan et al. (2024), the nutrient solution was prepared, and the pH was adjusted to 6.0. Foam culture plates with holes stabilised wheat seedlings in this hydroponic cultivation. In each plastic box, there were 20 seedlings. To ensure root healthy growth, oxygen pumps were used to supply oxygen to the nutrient solution. The nutrient solution was renewed every two days. When the third leaves were fully unfolded, wheat seedlings with similar growth conditions were picked out to carry out the following experiments.

In this study, 60 mg/L $CuSO_4$ (purity 98%, Beijing Suolaibao Technology Co. Ltd., Beijing, China) was chosen from three concentrations of $CuSO_4$ (30, 60 and 120 mg/L). Different amounts of $CuSO_4$ were weighed and then added to the nutrient solution. The pH of the $CuSO_4$ solution was also adjusted to 6.0. After 5 days of treatment, wheat plants treated with 120 mg/L $CuSO_4$ showed obvious leaf wilting and yellowing phenomena. However, wheat plants treated with 30 mg/L $CuSO_4$ showed no obvious leaf wilting and yellowing phenomenon. Meanwhile, wheat plants treated with 60 mg/L $CuSO_4$ showed only minor leaf wilting and yellowing phenomena. So, we chose 60 mg/L $CuSO_4$ to carry out this study. To explore the effect of Spd (purity 98%, Beijing Suolaibao Technology Co. Ltd., Beijing, China), 0.3, 0.9, and 1.8 mmol/L Spd were prepared by dissolving different weights of Spd in the nutrient solution. The pH of the Spd solution was also adjusted to 6.0. The roots of the three wheat groups were respectively firstly submerged in 500 mL 0.3, 0.9 and 1.8 mmol/L Spd contained in beakers for 8 h and then submerged in 500 mL 60 mg/L $CuSO_4$ or 500 mL nutrient solution contained in beakers for 10 days. For 60 mg/L $CuSO_4$ treatment, seedlings' roots were firstly submerged in 500 mL nutrient solution for 8 h and then submerged in 500 mL 60 mg/L $CuSO_4$ contained in beakers for 10 days. The roots of control plants were only submerged in 500 mL nutrient solution contained in 500 mL beakers for the same time as the above treatments. To keep the roots dark, all beakers were wrapped in aluminium foil. For each beaker, there were 12 seedlings for all treatments. Each treatment was repeated three times. For each replication, there were 12 seedlings. After 5 days of Cu treatment, top fully unfolded leaves were sampled, frozen by liquid N_2 and kept at –80 °C until analysis. After 10 days of Cu treatment, Cu contents and plant growth indicators were measured.

Assay of APX, GR, DHAR and MDHAR. According to Shan and Liang (2010), the extraction of APX, GR, DHAR and MDHAR was done, and their activities were measured. Leaves were grounded into a homogenate in 5 mL 50 mmol potassium phosphate (KH_2PO_4) buffer solution (pH 7.5) and then centrifuged to obtain the crude enzyme extract at 10 000 *g* for 20 min at 2 °C. Then, the crude enzyme extract was used to measure the activities of these enzymes on the spectrophotometer (UV759 model, Shanghai Jingke Scientific Instrument Co. Ltd., Shanghai, China). APX, GR, DHAR and MDHAR activities were analysed by detecting absorbance values at 290, 340, 340 and 265 nm wavelengths against the blank, respectively. The extinction coefficients 2.8, 6.2, 14.6 and 6.2 mmol/cm were used to calculate APX, GR, DHAR and MDHAR activities, respectively. Their specific activities were expressed as U/g fresh weight (FW).

Assay of GalLDH and γ-ECS. To measure GalLDH activity, leaves were homogenised in 5 mL 100 mmol KH_2PO_4 buffer (pH 7.4) and centrifuged at 300 *g* for 10 min at 2 °C. Then, the supernatant was continuously centrifuged at 10 000 *g* for 20 min at the same temperature. The sediment was suspended in KH_2PO_4 buffer and used to analyse GalLDH activity as Tabata et al. (2001) reported on the spectrophotometer (UV759 model, Shanghai Jingke Scientific Instrument Co. Ltd., Shanghai, China). The assay mixture included 0.2 mL enzyme solution, 2 mL 1.05 mg/mL cytochrome c (Cyt c) and 0.2 mL 56 mmol L-galactolactone (L-Gal). After L-Gal addition, the absorbance value at 550 nm wavelength was recorded against the blank. The calculation of GalLDH activity was done by using the extinction coefficient of 17.3 mmol/cm. To measure γ-ECS activity, leaves were grounded into a homogenate in 5 mL 100 mmol hydrochloric acid (HCl) and centrifuged to obtain the crude enzyme extract at 20 000 *g* for 10 min at 2 °C. The crude enzyme extract was used to analyse γ-ECS activity,

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as Ogawa et al. (2004) reported, by detecting the absorbance value at 660 nm wavelength against the blank. The γ-ECS activity was calculated using the extinction coefficient of 5.6 mmol/cm. Their specific activities were expressed as U/g FW.

Assay of AsA, GSH, AsA/DHA and GSH/GSSG. Leaves were grounded into a homogenate in 5 mL 5% trichloroacetic acid (TCA) and centrifuged at 12 000 *g* for 10 min at 2 °C. The supernatant was used to analyse AsA, DHA, GSH and GSSG contents. The contents of AsA and DHA were analysed as Hodges et al. (1996) reported by recording the absorbance value at 520 nm wavelength against the blank. The contents of AsA and DHA were calculated according to the standard curve. Ascorbate redox status was calculated as the ratio of its reduced form to oxidised form and expressed as AsA/DHA. The GSH and GSSG contents were analysed, as Griffith (1980) reported, by detecting the absorbance value at 412 nm wavelength against the blank. The contents of GSH and GSSG were calculated according to the standard curve. Glutathione redox status was calculated as the ratio of its reduced form to oxidised form and expressed as GSH/GSSG.

Assay of MDA and EL. To measure MDA content, leaves were homogenised in 5 mL KH_2PO_4 buffer (pH 7.8) and centrifugated at 12 000 *g* for 10 min at 4 °C. The supernatant was used to measure MDA content according to Hodges et al. (1999) by recording absorbance values at 532 nm and 600 nm wavelengths against the blank. The calculation of MDA content was done by using the extinction coefficient of 155 mmol/cm. To measure EL, the same number of fresh leaf discs were submerged in 20 mL deionised water at 25 °C for 3 h for each treatment. Then, the water solution's electrical conductivity (EC) was measured before and after boiling at 100 °C. Before and after boiling, EC was recorded as EC_0 and EC_1. EL was calculated as the percentage of EC_0 to EC_1.

Assay of Cu content in leaves and roots. The content of Cu in samples was measured as reported by Xu et al. (2011). As described in this method, all samples were washed with 10 mmol/L ethylenediaminetetraacetic acid (EDTA) to remove metals from the leaf and root surface. Leaves and roots were placed in the oven at 70 °C until the constant weight. Dry leaves and roots were digested by 10 mL of HNO_3 and $HClO_4$ (*v*/*v*, 3:1) at 95 °C. The digested residue was dissolved in 0.7 mL 1.0 mol/L HCl and then diluted with distilled water to 10 mL. Then, Cu content was analysed using flame atomic absorbance spectrometry (Hitachi 180-80, Tokyo, Japan) and calculated using the standard curve.

Measurement of photosynthetic performance. Chlorophyll (*Chl*) and carotenoid (Car) contents were measured, as reported by Song et al. (2016). Leaves were homogenised in 20 mL of 80% acetone and filtered using the funnel and filter paper. After filtration, the absorbance values at 665, 649 and 470 nm wavelengths were measured against the blank. Net photosynthetic rate (P_n) was detected through LI-COR 6400 photosynthesis system (Lincoln, Lincoln, USA). In the leaf chamber, the temperature, actinic light intensity and CO_2 concentration were respectively set to 26 °C, 1 200 μmol/m^2/s and 400 μmol/mol. Maximum photochemical efficiency of photosynthetic system II (F_v/F_m) was detected through a PAM-2500 chlorophyll fluorometer (Walz, Effeltrich, Germany). For dark adaptation, the leaves were covered by leaf clips for 30 min. Minimal fluorescence (F_0) and maximum fluorescence (F_m) were measured under dark adaptation. F_v/F_m was calculated as $(F_m - F_0)/F_m$ (Zai et al. 2021).

Assy of plant height and biomass. A meter ruler was used to measure the straight length from the base of the root to the top of wheat seedlings and recorded as plant height. Wheat biomass was measured by the drying method through an oven at 80 °C for 3 days until the constant weight. Then, the dry biomass was measured as wheat biomass.

Statistical analysis. Data presented in tables and figures were the average value of three replications. Through SPSS software 28.0.1.1 (Chicago, USA), means were compared by one-way analysis of variance, Duncan's multiple range test at the 5% level of significance, as well as Pearson correlation analysis. Excel Software 2019 (Redmond, USA) was used to organise data and draw tables and figures.

RESULTS

The selection of Spd concentration. Cu stress increased MDA, AsA and GSH contents compared to the control but lowered plant height (Table 1). Compared to Cu stress, all Spd concentrations plus Cu stress decreased MDA content and increased AsA and GSH contents and plant height. Spd alone also decreased MDA content, increased AsA and GSH contents and plant height against the control. Among three Spd concentrations, 0.9 mmol/L Spd had better effects on these indicators, which indicated that 0.9 mmol/L Spd could be used to elucidate how Spd enhanced wheat Cu tolerance.

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Table 1. Effects of different spermidine (Spd) concentrations on reduced ascorbate (AsA), reduced glutathione (GSH) and malondialdehyde (MDA) contents and plant height under copper (Cu) stress

Treatment	AsA	GSH	MDA	Plant height
	(μmol/g FW)		(nmol/g FW)	(cm)
Control	3.11 ± 0.20^{d}	0.42 ± 0.02^{f}	7.20 ± 0.49^{e}	24.70 ± 1.57^{b}
60 mg/L Cu	3.69 ± 0.20^{c}	0.55 ± 0.03^{cd}	18.82 ± 1.38^{a}	16.00 ± 1.00^{e}
0.3 mmol/L Spd	3.43 ± 0.16^{cd}	0.48 ± 0.02^{e}	6.36 ± 0.40^{f}	25.80 ± 1.34^{ab}
0.9 mmol/L Spd	3.95 ± 0.12^{bc}	0.60 ± 0.03^{c}	5.15 ± 0.27^{g}	28.10 ± 1.50^{a}
1.8 mmol/L Spd	3.70 ± 0.19^{c}	0.53 ± 0.03^{d}	5.87 ± 0.33^{f}	26.60 ± 1.33^{ab}
0.3 mmol/L Spd + 60 mg/L Cu	4.18 ± 0.22^{b}	0.67 ± 0.03^{b}	15.54 ± 1.07^{b}	18.40 ± 1.30^{d}
0.9 mmol/L Spd + 60 mg/L Cu	4.96 ± 0.30^{a}	0.89 ± 0.05^{a}	11.37 ± 0.66^{d}	21.00 ± 1.40^{c}
1.8 mmol/L Spd + 60 mg/L Cu	4.65 ± 0.25^{ab}	0.71 ± 0.04^{b}	13.02 ± 0.84^{c}	19.80 ± 1.40^{cd}

Wheat plants were firstly treated by Spd for 8 h and then exposed to 60 mg/L $CuSO_4$ or the nutrient solution for 5 days to measure AsA, GSH and MDA contents and for 10 days to measure plant height. Means with their standard deviations are in the table. Different small letters indicate significant differences ($P < 0.05$) among treatments. FW – fresh weight

Effects of Spd on enzymes in AsA and GSH recycling and biosynthetic pathway. Table 2 showed Cu stress-activated AsA and GSH recycling pathways by increasing APX, GR, DHAR and MDHAR activities against the control. When compared to Cu treatment, Spd plus Cu treatment further improved the enzyme activities of wheat seedlings. In contrast, Spd plus Cu stress increased APX, GR, DHAR and MDHAR activities to 4.75, 5.14, 3.77 and 2.96 U/g FW. Spd alone also enhanced these enzymes' activities against the control. These results implied that Spd enhanced AsA and GSH recycling pathways under Cu stress through the above four enzymes.

Table 2 also displayed Cu stress-activated AsA and GSH biosynthetic pathways by increasing GalLDH and γ-ECS activities against the control. Compared to Cu stress, Spd plus Cu stress increased above two enzymes' activities of wheat seedlings. In contrast, Spd plus Cu stress increased GalLDH and γ-ECS activities to 3.24 and 2.83 U/g FW. Spd alone also enhanced GalLDH and γ-ECS activities of wheat seedlings against the control. These results implied that Spd could also modulate AsA and GSH, the biosynthetic pathway through the above two key enzymes under Cu stress.

Effects of Spd on AsA and GSH contents, AsA/DHA and GSH/GSSG. In contrast with control, Cu stress enhanced the accumulation of AsA and GSH (Table 3). However, Cu stress decreased AsA/DHA and GSH/GSSG against the control. Under Cu stress, Spd further increased AsA and GSH accumulation, AsA/DHA and GSH/GSSG against Cu stress alone. In contrast, Spd plus Cu stress increased AsA content, GSH content, AsA/DHA and GSH/

Table 2. Effects of spermidine (Spd) on the activities of enzymes in reduced ascorbate (AsA) and reduced glutathione (GSH) recycling and biosynthetic pathway under copper (Cu) stress

Treatment	APX	GR	DHAR	MDHAR	GalLDH	γ-ECS
	(U/g FW)					
Control	2.32 ± 0.18^{d}	2.60 ± 0.14^{c}	1.60 ± 0.13^{c}	1.22 ± 0.11^{d}	1.35 ± 0.13^{c}	1.27 ± 0.10^{c}
0.9 mmol/L Spd	3.56 ± 0.20^{b}	3.47 ± 0.19^{b}	2.30 ± 0.15^{b}	1.97 ± 0.12^{b}	1.76 ± 0.14^{b}	1.80 ± 0.12^{b}
60 mg/L Cu	3.10 ± 0.21^{c}	3.75 ± 0.22^{b}	2.42 ± 0.17^{b}	1.70 ± 0.14^{c}	1.90 ± 0.17^{b}	1.68 ± 0.14^{b}
0.9 mmol/L Spd + 60 mg/L Cu	4.75 ± 0.28^{a}	5.14 ± 0.28^{a}	3.77 ± 0.21^{a}	2.96 ± 0.20^{a}	3.24 ± 0.21^{a}	2.83 ± 0.19^{a}

Wheat seedlings were firstly treated by Spd for 8 h and then exposed to 60 mg/L $CuSO_4$ or the nutrient solution for 5 days to measure these indicators. Means with their standard deviations are in the table. Different small letters indicate significant differences ($P < 0.05$) among treatments. APX – ascorbate peroxidase; GR – glutathione reductase; DHAR – dehydroascorbate reductase; MDHAR – monodehydroascorbate reductase; GalLDH – L-galactono-1,4-lactone dehydrogenase; γ-ECS – gamma glutamylcysteine synthetase; FW – fresh weight

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Table 3. Effects of spermidine (Spd) on reduced ascorbate (AsA) and reduced glutathione (GSH) contents, and AsA/dehydroascorbic acid (DHA) and GSH/oxidised glutathione (GSSG) ratios under copper (Cu) stress

Treatment	AsA	GSH	AsA/DHA	GSH/GSSG
	(μmol/g FW)			
Control	2.90 ± 0.18^{c}	0.38 ± 0.02^{c}	20.34 ± 1.32^{b}	22.15 ± 1.11^{b}
0.9 mmol/L Spd	3.70 ± 0.20^{b}	0.55 ± 0.03^{b}	23.60 ± 1.20^{a}	26.00 ± 1.47^{a}
60 mg/L Cu	3.57 ± 0.23^{b}	0.49 ± 0.04^{b}	7.85 ± 0.44^{d}	9.24 ± 0.53^{d}
0.9 mmol/L Spd + 60 mg/L Cu	4.62 ± 0.29^{a}	0.78 ± 0.06^{a}	14.60 ± 0.89^{c}	15.97 ± 0.80^{c}

Wheat plants were firstly treated by Spd for 8 h and then exposed to 60 mg/L $CuSO_4$ or the nutrient solution for 5 days to measure these indicators. Means with their standard deviations are in the table. Different small letters indicate significant differences ($P < 0.05$) among treatments. FW – fresh weight

GSSG to 4.62 μmol/g FW, 0.78 μmol/g FW, 14.60 and 15.97. Compared to control, Spd alone also increased ascorbate and glutathione's reduced forms and redox state. These results implied that Spd improved the antioxidant capacity by increasing AsA and GSH contents and maintaining the redox homeostasis under Cu stress.

Effects of Spd on MDA and EL. Compared to the control, Cu stress increased MDA content and EL (Figure 1). Spd plus Cu stress declined MDA and EL levels compared to Cu stress. Spd plus Cu stress reduced MDA content and EL to 11.68 nmol/g FW and 17.00% compared to Cu alone. Spd alone also decreased these indicators of wheat seedlings against the control. Our results illustrated that Spd stimulated wheat Cu tolerance.

Effects of Spd on Cu content. Compared with the control, Cu stress promoted Cu accumulation in plant tissues (Figure 2). Compared with Cu stress, Spd plus Cu stress reduced the accumulation of Cu. Compared with Cu stress, Spd plus Cu stress lowered Cu contents in leaves and roots to 68.8 μg/g DW and 152.9 μg/g DW. Spd alone did not significantly influence Cu contents in tissues against the control. These results implied that Spd could reduce Cu accumulation in tissues of Cu-stressed wheat seedlings.

Effects of Spd on the photosynthetic performance. Compared to the control, Cu stress decreased P_n, F_v/F_m, and pigments *Chl* and Car contents (Table 4). Compared to Cu stress, Spd plus Cu stress respectively increased P_n, F_v/F_m, and pigments *Chl* and Car contents to 15.22 μmol/m^2/s, 0.74, 1.55 mg/g FW and 0.38 mg/g FW. Compared to the control, Spd alone also increased P_n, F_v/F_m, and the contents of the above two pigments. These results implied that Spd enhanced wheat photosynthetic performance under Cu stress.

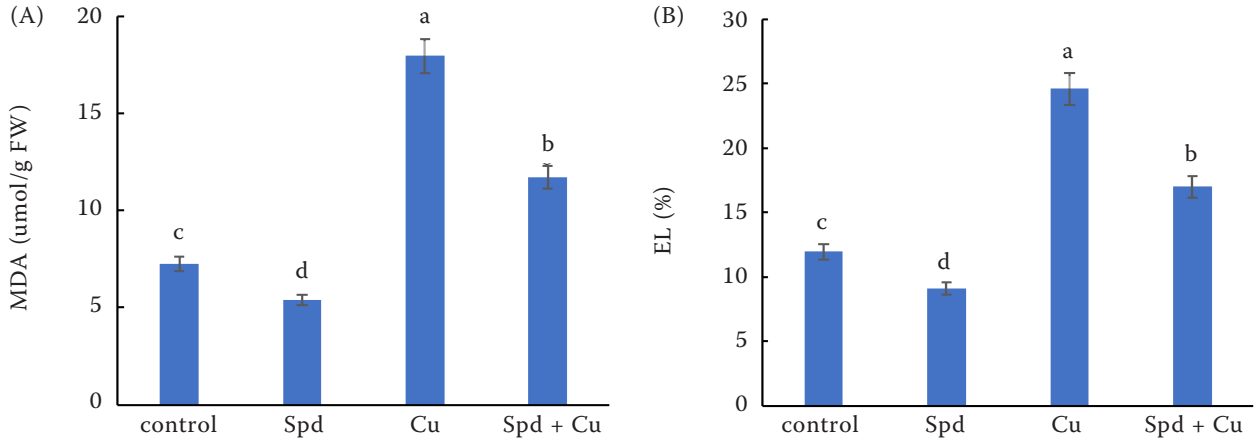

Figure 1. Effects of spermidine (Spd) on malondialdehyde (MDA) content and electrolyte leakage (EL) under copper (Cu) stress. Wheat plants were firstly treated as below. Control – half-strength Hoagland's solution; Cu – 60 mg/L $CuSO_4$; Spd + Cu – 0.9 mmol/L Spd + 60 mg/L $CuSO_4$. Wheat seedlings were treated by Spd for 8 h and then exposed to 60 mg/L $CuSO_4$ or the nutrient solution for 5 days to measure these indicators. Means with their standard deviations are in the figure. Different small letters indicate significant differences ($P < 0.05$) among treatments. FW – fresh weight

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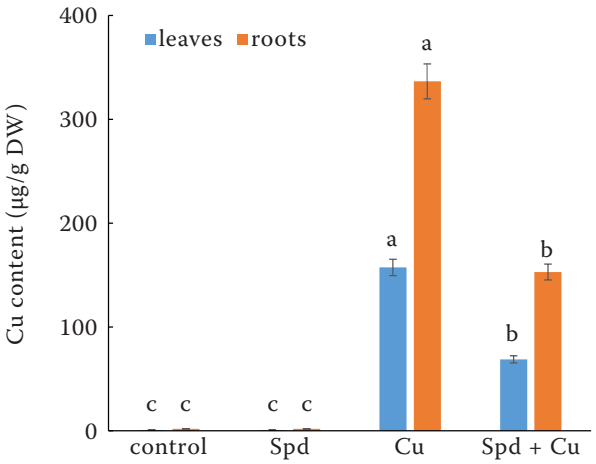

Figure 2. Effects of spermidine (Spd) on leaves and roots' copper (Cu) content. Wheat seedlings were treated as below. Control – half-strength Hoagland's solution; Cu – 60 mg/L $CuSO_4$; Spd + Cu – 0.9 mmol/L Spd + 60 mg/L $CuSO_4$. Wheat plants were firstly treated by Spd for 8 h and then exposed to 60 mg/L $CuSO_4$ or the nutrient solution for 10 days. Means with their standard deviations are in the figure. Different small letters indicate significant differences ($P < 0.05$) among treatments. DW – dry weight

Effects of Spd on growth parameters. Compared to control, Cu stress decreased plant height and dry biomass (Figure 3). Compared to Cu stress, Spd plus Cu stress respectively increased plant height and dry biomass to 20.2 cm and 123.8 mg/plant. Spd alone also increased these growth indicators against the control. These results directly implied that Spd enhanced the Cu tolerance of wheat seedlings.

Pearson correlation analysis among the individual parameters. We analysed the relationships among different tested parameters by using Pearson correlation analysis. The results showed that plant height and biomass had significant positive relationships with the photosynthetic parameters, including *Chl* content, Car content, F_v/F_m and P_n. These results indicated that plant growth was closely related to photosynthetic performance. However, plant growth and photosynthetic parameters all had significant negative relationships with MDA content, EL and the contents of Cu in leaves and roots. These results indicated that Cu stress induced Cu accumulation in plant tissues and caused oxidative stress to wheat crops, further inhibiting wheat growth and photosynthetic performance. Moreover, the results illustrated that AsA and GSH contents had significant positive relationships with the activities of enzymes in AsA and GSH recycling and biosynthesis, which proved again that AsA and GSH contents had close relationships with these enzymes. However, this study showed that growth and photosynthetic parameters did not have positive relationships with AsA and GSH contents but had significant positive relationships with AsA/DHA and GSH/GSSG. This phenomenon illustrated that the ascorbate and glutathione redox state determined plant growth and photosynthetic performance, not the absolute contents of AsA and GSH. Meanwhile, we showed that MDA content, EL and the contents of Cu in leaves and roots had positive relationships with AsA and GSH contents and their recycling and biosynthetic enzymes, which indicated that Cu-induced oxidative stress caused the upregulation of AsA and GSH recycling and biosynthetic metabolism under Cu stress. As shown in Tables 2–4 and Figures 1–3, Spd decreased MDA content, EL, Cu accumulation, and improved parameters related to the photosynthetic performance, the ascorbate and glutathione metabolism and plant growth. Thus, the above results of Pearson correlation analysis illustrated that Spd enhanced wheat growth under Cu stress by improving photosynthetic performance and redox homeostasis.

Table 4. Effects of spermidine (Spd) on net photosynthetic rate (P_n), maximum photochemical efficiency of photosynthetic system II (F_v/F_m), and chlorophyll (*Chl*) and carotenoids (Car) contents under copper (Cu) stress

Treatment	*Chl* (mg/g FW)	Car (mg/g FW)	F_v/F_m	P_n (µmol/m²/s)
Control	1.78 ± 0.14^b	0.49 ± 0.04^b	0.80 ± 0.02^b	18.75 ± 1.21^b
0.9 mmol/L Spd	2.06 ± 0.12^a	0.57 ± 0.04^a	0.86 ± 0.03^a	21.20 ± 1.10^a
60 mg/L Cu	1.26 ± 0.09^d	0.23 ± 0.02^d	0.65 ± 0.02^d	10.40 ± 0.78^d
60 mg/L Cu + 0.9 mmol/L Spd	1.55 ± 0.11^c	0.38 ± 0.03^c	0.74 ± 0.02^c	15.22 ± 1.01^c

Wheat plants were firstly treated by Spd for 8 h and then exposed to 60 mg/L $CuSO_4$ or the nutrient solution for 5 days. Means with their standard deviations are in the table. Different small letters indicate significant differences ($P < 0.05$) among treatments. FW – fresh weight

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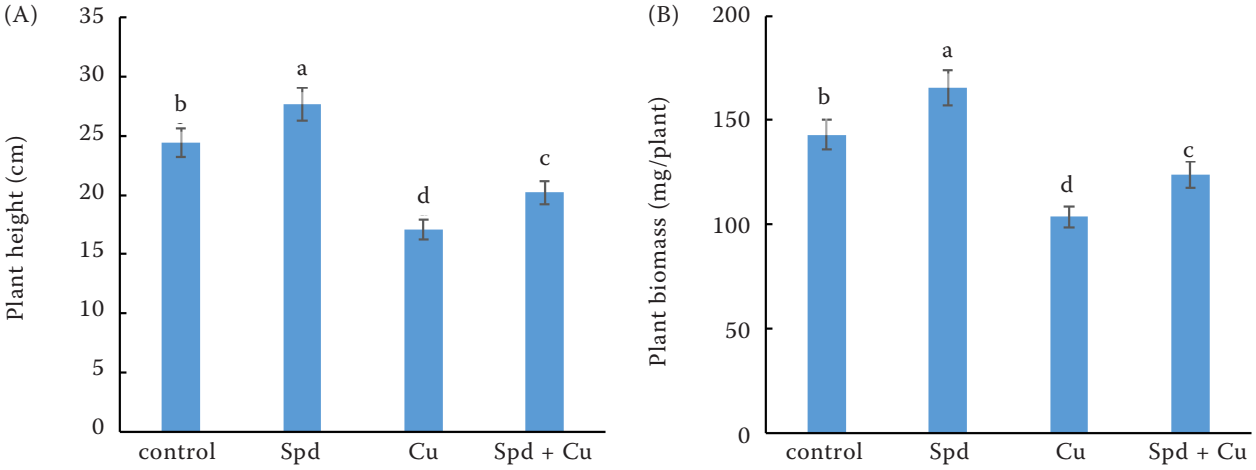

Figure 3. Effects of spermidine (Spd) on plant height and biomass under copper (Cu) stress. Wheat plants were treated as below. Control – half-strength Hoagland's solution; Cu – 60 mg/L $CuSO_4$; Spd + Cu – 0.9 mmol/L Spd + 60 mg/L $CuSO_4$. Wheat seedlings were firstly treated by Spd for 8 h and then exposed to 60 mg/L $CuSO_4$ or the nutrient solution for 10 days. Means with their standard deviations are in the figure. Different small letters indicate significant differences ($P < 0.05$) among treatments

DISCUSSION

Increasing research reported that Cu stress induced peroxide damage to plants. The current study showed significant positive relationships between MDA and EL levels and Cu contents in plant tissues, indicating that Cu stress induced peroxide damage to wheat seedlings. Previous research also demonstrated that plants decreased the level of oxidation stress caused by Cu stress by increasing GR and APX activities and GSH and AsA contents (Wang et al. 2011). Our research also found that Cu stress increased these indicators in wheat leaves. Moreover, we uncovered that Cu stress boosted the activities of other enzymes in AsA and GSH recycling and biosynthetic pathways, including DHAR, MDHAR, GalLDH and γ-ECS in wheat seedlings. We revealed that AsA and GSH contents had significant positive relationships with these enzymes' activities through Pearson correlation analysis. Meanwhile, we displayed that MDA content, EL and the contents of Cu in leaves and roots had positive relationships with AsA and GSH contents and their recycling and biosynthetic enzymes, which indicated that Cu-induced oxidative stress caused the upregulation of AsA and GSH recycling and biosynthetic metabolism under Cu stress. Therefore, this study indicated that wheat seedlings could decrease the oxidation stress caused by Cu stress by enhancing AsA and GSH recycling and biosynthetic pathways.

Only a few studies have shown that Spd reduced peroxide damage in Cu-stressed plants (Xu et al. 2011, Agami 2016). Furthermore, there is very little knowledge of the role of Spd in regulating ascorbate and glutathione metabolism in Cu-stressed plants. As we all know, AsA is important in defending against stress (Khafagy et al. 2009). In plant cells, AsA content is regulated by its recycling and biosynthetic metabolism-related enzymes, including APX, DHAR, MDHAR and GalLDH. Agami (2016) documented that Spd increased APX activity in Cu-stressed wheat seedlings. As APX is one important enzyme for the recycling pathway of ascorbate, previous studies indicated that Spd could regulate AsA content through its recycling metabolism. In this research, our findings showed that Spd increased APX activity in Cu-stressed wheat seedlings, which was similar to the findings of Agami (2016). Besides, our findings also indicated that Spd increased the activities of circulatory metabolism-related enzymes DHAR and MDHAR. Moreover, we found that Spd enhanced the anabolic metabolism-related enzyme GalLDH activity under Cu stress. Meanwhile, the findings showed that Spd increased AsA content under Cu stress. Through Pearson correlation analysis, we found that AsA content had significant positive relationships with APX, DHAR, MDHAR and GalLDH activities. Hence, Spd enhanced AsA regeneration *via* APX, MDHAR, DHAR, and AsA biosynthesis *via* GalLDH under Cu stress. Current results implied that Spd enhanced wheat Cu tolerance by modulating AsA recycling metabolism and biosynthesis, which is one novel for this study.

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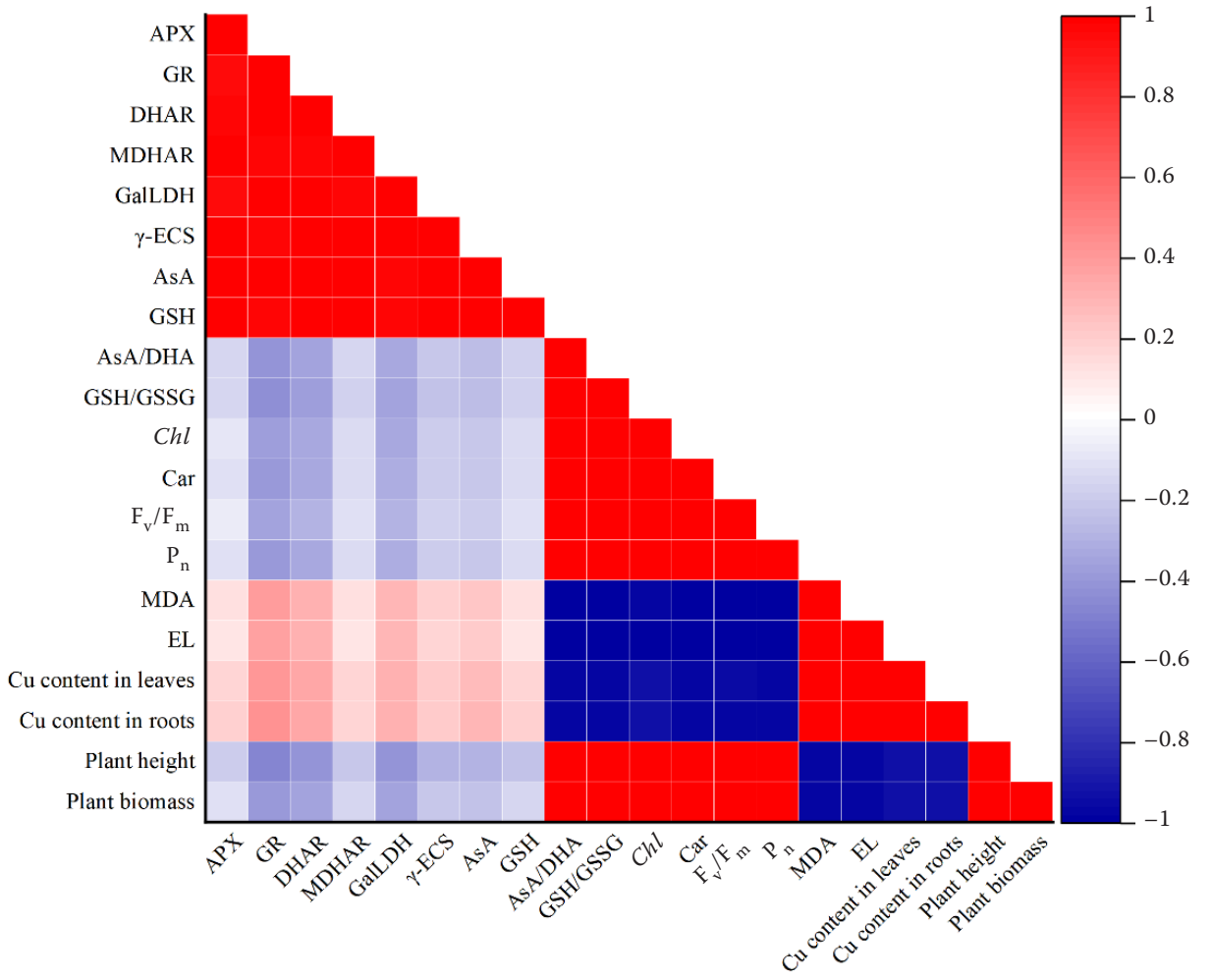

Figure 4. Pearson correlation analysis among the individual parameters. APX – ascorbate peroxidase; GR – glutathione reductase; DHAR – dehydroascorbate reductase; MDHAR – monodehydroascorbate reductase; GalLDH – L-galactono-1,4-lactone dehydrogenase; γ-ECS – gamma glutamylcysteine synthetase; AsA – reduced ascorbate; GSH – reduced glutathione; AsA/DHA – reduced ascorbate/dehydroascorbic acid; GSH/GSSG – reduced glutathione/oxidised glutathione; *Chl* – chlorophyll; Car – carotenoid; F_v/F_m – maximum photochemical efficiency of PSII; P_n – net photosynthetic rate; MDA – malondialdehyde; EL – electrolyte leakage

In plant cells, GSH content is regulated by γ-ECS and GR, respectively responsible for GSH recycling and biosynthetic pathway. However, there is no report on the effects of SPD on γ-ECS and GR activities in Cu-stressed wheat crops. For our study, the findings displayed that Spd modulated the recycling pathway of glutathione by increasing GR activity and the biosynthesis of glutathione by increasing γ-ECS activity under Cu stress. Meanwhile, we found that Spd improved GSH content under Cu stress. Through Pearson correlation analysis, we found that GSH content had significant positive relationships with the activities of GR and γ-ECS. Hence, Spd enhanced GSH regeneration *via* GR and GSH biosynthesis *via* γ-ECS Cu-stressed wheat seedling leaves. Current results implied that Spd enhanced wheat Cu tolerance by modulating both the recycling metabolism and biosynthesis of glutathione, which is another novel for this study.

It has been reported that many stresses destroyed plant redox homeostasis by inducing oxidative damage. In plants, ascorbate and glutathione redox states contribute more to redox homeostasis, especially under stress. Ascorbate and glutathione redox states were expressed as AsA/DHA and GSH/GSSG, respectively. For this reason, AsA/DHA and GSH/GSSG are more important than AsA and GSH contents for plant defence against stresses. It has

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been documented that many stresses destroyed the redox homeostasis by decreasing AsA/DHA and GSH/GSSG ratios. Fardus et al. (2023) found that Cu stress reduced lentils' GSH/GSSG ratio. This study uncovered that Cu stress decreased wheat AsA/DHA and GSH/GSSG. Through Pearson correlation analysis, we found that AsA/DHA and GSH/GSSG had significant negative relationships with MDA content, EL and Cu contents in leaves and roots, which proved that Cu stress induced the peroxide damage and destroyed the redox homeostasis of wheat seedlings. Increasing research displayed that Spd alleviated the peroxide damage under drought, salt, Cu and cadmium stresses (Yang et al. 2011, Xu et al. 2011, Yin et al. 2014, Wu et al. 2020). However, the effect of Spd on AsA/DHA and GSH/GSSG under Cu stress is still unclear. The current study showed that Spd could increase wheat AsA/DHA and GSH/GSSG under Cu stress. Through Pearson correlation analysis, we found that plant growth and photosynthetic parameters had significant positive relationships with AsA/DHA and GSH/GSSG. Meanwhile, AsA/DHA and GSH/GSSG had significant negative relationships with MDA content, EL and Cu contents in leaves and roots. Therefore, it is the first time to report that Spd could alleviate Cu stress-induced peroxide damage of wheat seedlings by modulating the redox homeostasis *via* increasing AsA/DHA and GSH/GSSG, which provides more information for its application in agriculture. However, AsA/DHA and GSH/GSSG did not have positive relationships with AsA and GSH contents and their recycling and biosynthetic enzymes. This phenomenon illustrated that AsA and GSH contents and their recycling and biosynthetic enzymes were not the only factors for the mechanism of Spd in regulating AsA/DHA and GSH/GSSG under Cu stress. Therefore, the mechanism by which Spd regulates AsA/DHA and GSH/GSSG was very complicated. As reported, DHA content had a close relationship with ascorbate oxidase (AO) activity, and GSSG content had a close relationship with glutathione peroxidase (GPX). So, exploring the effects of Spd on AO and GPX activities in Cu-stressed wheat leaves is interesting and can provide more information to elucidate how Spd regulates AsA/DHA and GSH/GSSG under Cu stress.

Previous research showed that Cu stress reduced plant growth (Gong et al. 2021, Alshegaihi et al. 2024, Fardus et al. 2023). Meanwhile, Cu stress inhibited plant growth due to Cu over-accumulation in plant tissues (Gong et al. 2021, Saman and Sepehri 2021). The current study found that plant growth parameters had significant negative relationships with Cu contents in leaves and roots, indicating that Cu stress had similar effects on wheat growth parameters, plant height and biomass. This study also revealed that Spd lowered Cu accumulation in leaves and roots, improving wheat height and biomass. Therefore, our results illustrated that Spd promoted plant growth of wheat seedlings by reducing Cu accumulation in wheat seedlings, which provides more information for its use in agriculture.

As is well known, chloroplasts are responsible for plant photosynthesis. In chloroplast, photosystem I and II produce ROS under light, mainly including $O_2^{\cdot -}$, H_2O_2 and $^1O_2$ (Castro et al. 2021). Chloroplasts have corresponding antioxidant systems to detoxify these ROS. In the photosystem I, $O_2^{\cdot -}$ can be dismutated by SOD or spontaneously formed into H_2O_2 in the matrix of the thylakoid membrane. H_2O_2 is also detoxified by POD, CAT and enzymes in the AsA-GSH cycle. We revealed that Spd enhanced the capacity of wheat seedlings to scavenge H_2O_2 by improving the activities of enzymes in the AsA-GSH cycle. Whereas, we did not investigate the effects of Spd on SOD, POD and CAT activities in leaves of wheat cultivar Yuanfeng16 under Cu stress. Thus, it will be interesting to carry out this work to elucidate the role of Spd in detoxifying the excess ROS under Cu stress. In photosystem II, $^1O_2$ can be cleared by reacting with other molecules, such as Car (Ramel et al. 2012). This study revealed that Spd increased Car content under Cu stress. These findings indicated that Spd could enhance wheat capacity to clear $^1O_2$ by promoting Car content. In these ways, Spd protected the photosynthetic apparatus of wheat seedlings against Cu-induced peroxide damage. We also showed that Spd increased *Chl* content under Cu stress. Car and *Chl* play vital roles in light absorption and utilisation as main photosynthetic pigments. Therefore, this study illustrated that Spd enhanced wheat's capacity to absorb and utilise light energy by promoting the contents of Car and *Chl*. Meanwhile, we showed that Spd could increase wheat F_v/F_m under Cu stress. Thus, this study manifested that Spd could improve wheat light energy utilisation rate under Cu stress. We found significant positive relationships among *Chl* content, Car content, P_n and F_v/F_m through Pearson correlation analysis. Based on the above research results, our study clearly indicated that Spd enhanced wheat photosynthetic performance under Cu stress.

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Our findings indicated that Spd modulated the ascorbate and glutathione metabolism by increasing APX, GR, MDHAR, DHAR, GalLDH and γ-ECS activities, and AsA and GSH contents, as well as AsA/DHA and GSH/GSSG ratios, which further enhanced the antioxidant ability and maintained the redox homeostasis. Meanwhile, Spd also reduced Cu accumulation in wheat leaves and roots. This way, Spd improved wheat photosynthetic performance and growth under Cu stress. Current findings displayed new knowledge of the physiological mechanism of Spd in modulating wheat Cu tolerance. They supplied more information for the potential application of Spd in agricultural management to control the growth of wheat crops planted in Cu-polluted soil. Although the price of Spd is not cheap at present, the results of this study at least demonstrated that Spd can be applied as a new potential exogenous chemical to mitigate wheat Cu toxicity, especially when its price is significantly reduced in the future. However, our research only investigated the role of Spd in enhancing wheat Cu tolerance at the third leaf stage. Therefore, extending this to other developmental stages of wheat crops will be more useful, such as the tillering stage, jointing stage and heading stage. These further studies will provide enough evidence for Spd application in the future production of wheat crops planted in Cu-polluted soil.

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